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Title: Honey bees and neonicotinoid-treated corn seed: contamination, exposure, and effects

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Abstract (250 words max)

Most corn (*Zea mays*) seeds planted in the US in recent years are coated with a seed treatment containing one of two neonicotinoid insecticides: clothianidin or thiamethoxam. Abrasion of the seed coating generates insecticide-laden dust that disperses through the landscape during corn planting and has resulted in a number of 'bee-kill' incidents in North America and Europe. We conducted field and semi-field experiments to investigate the linkage between corn-planting and honey bee mortality in a landscape dominated by corn agriculture. For three years, we consistently observed increased presence of corn seed treatment insecticides in bee-collected pollen and elevated worker bee mortality that coincided with corn-planting. Pollen contamination with corn seed treatment insecticides positively correlated with cornfield area surrounding the apiaries. Additionally, significantly increased worker mortality was observed in experimental colonies fed with bee-collected pollen with known contamination of corn seed treatment insecticides. Deliberative Process / Ex. 5

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Deliberative Process / Ex. 5 Exposure to seed treatment neonicotinoids during corn planting has clear short-term detrimental effects on honey bee colonies being kept in corn growing areas and has the potential to seriously affect the economic viability of beekeeping operations that are dependent on maximizing honey bee populations in the springtime.

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Keywords: clothianidin, thiamethoxam, *Apis mellifera*, *Zea mays*, pollinators, seed treatment

Introduction

It is estimated that 90% of corn (*Zea mays*) in the United States is grown from seed treated with neonicotinoid insecticides. The predominant neonicotinoids used in corn seed treatments are clothianidin (Poncho®, EPA Registration Number 264-789) and thiamethoxam (Cruiser®, EPA Registration Number 100-1208) at rates between 0.125 to 1.25 mg active ingredient per seed, depending on the corn insect pest of concern (Douglas and Tooker 2015). Assuming a seeding rate of 54,000 – 82,000 seeds per hectare (Thomison 2015), up to 100 g/hectare of insecticide active ingredients are applied to sown fields each year. These broad-spectrum insecticides are highly toxic to many insects, including honey bees (*Apis mellifera* L.), to which they are lethal in nanogram quantities -- as low as 0.003 μg/bee for oral LD_{50} and 0.02 μg/bee for contact LD_{50} over 48 hr (Decourtye and Devillers 2010, Laurino et al. 2013).

A link between observations of honey bee mortality and the planting of neonicotinoid-treated corn seeds was suspected as early as the late 1990s, when researchers in Italy noted a rise in colony damage reports coinciding with spring corn planting (Bortolotti et al. 2009). In subsequent years, similar patterns of honey bee mortality were observed in Italy (Schnier et al. 2003, Greatti et al. 2006, Bortolotti et al. 2009), France (Giffard and Dupont 2009), and Slovenia (Alix et al. 2009, van der Geest 2012, Žabar et al. 2012). In 2008, a large-scale bee kill in Germany and neighboring parts of France was attributed to the planting of neonicotinoid-treated corn after an extensive investigation found neonicotinoid residues in dead bees, bee bread, and plant samples collected from the affected area (Forster 2009, Nikolakis et al. 2009, Pistorius et al. 2009, Chauzat et al. 2010). Since then, additional incidents of honey bee mortality during corn planting have been reported in Slovenia and neighboring Hungary (van der Geest 2012), the United States (Krupke et al. 2012; L. Keller, personal communication, 2016) and Canada (Health Canada 2013).

While these reports clearly establish a link between honey bee mortality and the planting of corn with a neonicotinoid seed treatment, the route through which bees are exposed to lethal levels of seed treatment insecticide is difficult to ascertain. During the planting process, seed treatment material sloughs off the seed surface in small particles that are available to disperse in the environment (**Fig. 1**). Bees may encounter these particles as dust deposited on flowers [HYPERLINK "https://paperpile.com/c/DPSqGI/fTCC3" \h], nectar and pollen contamination via uptake from the soil [HYPERLINK "https://paperpile.com/c/DPSqGI/j6Rho" \h], contamination of surface water [HYPERLINK "https://paperpile.com/c/DPSqGI/51oeC+GzPEy" \h], contamination of guttation fluids [HYPERLINK "https://paperpile.com/c/DPSqGI/Fmet6" \h], and in-flight contact with aerial dust [HYPERLINK "https://paperpile.com/c/DPSqGI/GkQu2" \h]. Soil containing insecticide residues left over from previous years of planting may also become airborne during planting and contribute to bee exposure during this period [HYPERLINK "https://paperpile.com/c/DPSqGI/ro7qb" \h]. While each of these routes may contribute to honey bee exposure, identifying the most significant route or routes is crucial for designing mitigation strategies.

This study aims to evaluate the exposure of honey bee colonies to corn seed treatment neonicotinoids via incorporation of corn seed treatment dust particles into pollen loads brought back to the hive by foraging workers. To better understand the association between corn planting, neonicotinoid residues in honey bee-collected pollen, and honey bee mortality, we conducted a three-year field study (2013 - 2015) in Ohio, one of largest corn-growing states in the United States. Each year, we measured worker mortality and pesticide contamination of bee-collected pollen prior to, during, and after corn planting. In 2015, we expanded the investigation to examine pollen contamination and bee mortality in apiaries located in landscapes with varying intensities of corn agriculture. We also analyzed neonicotinoid residues in stored pollen to evaluate the persistence of corn seed treatment insecticides inside honey bee colonies, and monitored the growth and overwinter survival of the colonies after exposure. To experimentally test the link between contamination of pollen and worker bee mortality, a semi-field experiment was conducted in which colonies in a controlled environment were provisioned with pollen collected during corn-planting containing known levels of neonicotinoid residues.

Materials and Methods

Study sites

A total of 13 apiaries located throughout the corn-growing region of central Ohio were monitored prior to, during, and after corn-sowing from late-April until the end of May in 2013 (3 apiaries), 2014 (6 apiaries) and 2015 (10 apiaries). Four apiaries (CH, MB, FSR, WB) were studied in multiple years (Supplemental Material S1). Apiaries were located at least 4 km from each other and were selected to represent a range of agricultural intensity, including one suburban apiary in 2015 with minimal corn agriculture within foraging range. Apiaries consisted of between 4 - 20 colonies. Two to four healthy, actively foraging colonies, varying in sizes and queen ages, were monitored for worker mortality (see Supplemental Material S1 for colony information). All colonies were housed in eight- or ten-framed Langstroth hives.

The timing of corn planting was identified through direct observation of planter activity near apiary sites and communication with farmers. The bulk of sowing activity in the study area occurred between May 5 – 16 in 2013, May 5 – 10 in 2014, and May 2 – 8 in 2015. These dates were in concordance with state-wide agricultural statistics for each year (USDA National Agricultural Statistics Service). Less intensive corn planting continued beyond this period in all years, but was particularly drawn out in 2014 when high rainfall resulted in planting and re-planting activity through the end of May.

Landscape characterization

The landscape surrounding each apiary was characterized within a 2-km radius around the apiary. Visual ground-truthing supplemented by satellite imagery (Google OpenLayers), was used to classify landscapes into crop field, forest, treeline, herbaceous strips in field margins and roadsides, and residential lots. Crop type was determined by a second visual inspection in early summer and the USDA crop data layer [HYPERLINK "https://paperpile.com/c/DPSqGI/9oCJ6" \h]. All landscape data were analyzed and visualized using QGIS software [HYPERLINK "https://paperpile.com/c/DPSqGI/om7Ly" \h]. Apiaries in 2013 and 2014 were surrounded by a high proportion of cornfields within 2 km of the apiaries, ranging from 31 – 45% corn in 2013 and 21 – 51 % in 2014. In 2015 there was a wider gradient of corn area was in the foraging range of apiaries, 0 - 49% corn.

Sampling and pesticide screening

Pollen pellets carried on the pollen baskets of foraging bees returning the colonies were harvested using bottom-mounted pollen traps (Sundance I, Ross Rounds, Inc. Canandaigua, New York, USA) installed on two strong hives at each apiary. Trapped pollen was collected every 2 –

4 days, pooled by sites and stored in darkness at -20 °C until further analyses.

In 2015, pollen and nectar stored by honey bees were also analyzed to evaluate the persistence of neonicotinoid residues inside the hives. Honey and bee bread (compacted pollen in comb cells) were sampled from cells peripheral to the brood area where bees were actively depositing food. Samples were collected from two queen-right colonies at seven apiaries (DS, SC, BR, HR, TV, MO) during four sampling periods: before planting (April 27 – 30), during planting (May 5 – 7), immediately after planting (May 12 – 13), and two weeks after planting (May 20 – 22). All samples were stored in darkness at -20 °C until further analyses.

Five grams of pollen from each site and sampling date were extracted using a modified QuEChERS protocol for the 2013 - 2014 samples (Camino-Sanchez et al. 2010). Pollen, honey and bee bread from 2015 were extracted from 1 – 5 g of sample following a method by Yáñez et al. (2014) except ethyl acetate was used instead of dichloromethane. In all years extracts were analyzed for neonicotinoid insecticides (clothianidin, thiamethoxam and imidacloprid) using liquid chromatography tandem mass spectrometry (LC-MS-MS) methods. Analysis was performed by the USDA-AMS lab in Gastonia, North Carolina (2013 - 2014 samples) and EPA National Exposure Research Laboratory in Athens, Georgia (2015 samples). All residues were reported as mass-mass concentration (µg/kg).

Dead bee trapping

Under-basket style dead bee traps (102x51x15 cm; Human et al. 2013) were placed in front of the monitored colonies from late April until end of May or early June, 1 – 2 weeks after corn planting activities had ceased. Dead bees in traps were counted and traps were emptied every 2 – 4 days.

Statistical analyses: worker mortality and pollen contaminations

The number of bees counted in dead bee traps on each visit was averaged over the number of days elapsed since the last visit to estimate daily dead bee counts for each colony. Because large colonies eject more dead bees than small colonies, a daily mortality index, denoted by M_i, was calculated using the following formula:

$$M_i = N_i / N_{max} \qquad \text{(Equation 1)}$$

where
N_i is the number of dead bees per day on a given date i
N_{max} the highest number of dead bees per day of a given colony observed during the sampling period

The standardized mortality M_i ranges between 0 and 1 for each colony unless $N_{max} = 0$. In an unlikely event when no dead bee was observed in the trap, $M_i = 0$. If dead bee counts were greater than 0 and consistent throughout the sampling period, i.e. N_i values did not deviate much from N_{max}, then all M_i values would be near 1 regardless of the corn-planting intensity.

To compare bee mortality between planting and non-planting periods, the means of M_i values for each period were calculated for each colony. Means of the same colony were then compared using paired-sample t-tests. A separate analysis was performed for each year.

The mortality rate data represent a time series with a known intervention (i.e., corn planting). The intervention effects and mortality-insecticide relations can be tested simultaneously using time series intervention analysis. Let M_t denote the mortality rate at time t and X_t denote the insecticide concentration at time t for a given site-year-colony. The test for differences in mortality rate between the planting and non-planting periods can be modeled by the following time series intervention model:

$$M_t = \beta_0 + \beta_1 * I(t \text{ in planting period}) + e_t \qquad \text{(Equation 2)}$$

where I(t in planting period) = 1 if t is in planting period and =0 otherwise. The model parameter β_1 is the difference in mortality rate between the planting and non-planting periods and represents a step intervention. The null hypothesis, H_0: β_1=0, can be tested using a likelihood-based test statistic for a time series model from the Box-Jenkins class of ARMA models. The test for a linear association between M_t and X_t can be parametrized as a time series regression model:

$$M_t = \beta_0 + \beta_2 * X_t + e_t \qquad \text{(Equation 3)}$$

The hypothesis H_0: β_2=0 is equivalent to zero correlation between mortality rate and insecticide concentration. We can combine Equations 1 & 2 into a time series intervention model

$$M_t = \beta_0 + \beta_1 * I(t \text{ in planting period}) + \beta_2 * X_t + e_t \qquad \text{(Equation 4)}$$

The time series intervention model can be generalized to allow for more general types of interventions (i.e., pulse, step, trend) and multiple predictor variables including lagged values of insecticide concentrations. The time series intervention model with a first-order autoregressive structure is

$$M_t - \mu_t = \rho(M_{t-1} - \mu_{t-1}) + e_t$$
$$\mu_t = \beta_0 + \beta_1 * X1_t + \beta_2 * X2_t + \ldots \beta_p * Xp_t \qquad \text{(Equation 5)}$$

where the predictor variables are either 0-1 indicator variables or continuous variables, possibly lagged values of one or more insecticide concentrations. M_t is assumed to be a stationary autoregressive-moving average time series. The time series intervention analysis provides more information than the paired t-test and correlation analysis. The mortality rate time series for each colony at an apiary site is assumed to be an independent representation of a time series. The time series model with intervention is fit by site and year. Data can be combined for either different years at the same site or different sites for the same year to test the adequacy of a common model. Because the insecticide concentration data are most complete for 2013 and 2015, the time series intervention model can be used to analyze the mortality rate data for 2013 and 2015 but not 2014.

Mortality index data for 2013 and 2015 were analyzed using time series intervention model.

Post-planting colony growth

To address the question of whether exposure to corn seed treatment insecticides in May is linked to long-term consequences in colony growth, we tracked the colonies in 2015 from April through March 2016. Four detailed colony inspections were performed using a modified Liebfelder method (Delaplane et al. 2013) on April 28 – 30 (before planting), May 20 – 22 (after planting), June 19 - 24, and August 14 - 19. During the inspection each frame from the monitored colonies was examined to record the area of coverage with the following components: adult bees, brood (open and capped), pollen, and honey. Additionally, the total adult bee population was estimated by looking up and down frame spaces to estimate "seams" of bees. All colonies were managed using standard beekeeping practices. *Varroa destructor* mites were controlled by applying formic acid (Mite Away Quick Strip, NOD Apiary Products) in June and vaporized oxalic acid in November. Plain baker's fondant (Dawn Food Products, Inc., Jackson, MI, USA) and Dadant AP23 winter patties (Dandant & Sons Inc., Hamilton, IL, USA) were fed to the colonies, as needed, through the winter. The number of surviving colonies was recorded on March 24, 2016.

We examined whether the relative change in each colony variable through time was associated with neonicotinoid concentrations measured in pollen in May. Relative change for each variable was calculated as:

$$Relative\ change\ (\%) = \frac{final - initial}{initial} * 100$$

We considered each interval between inspection dates, as well as the interval between the first and last inspections. To determine whether neonicotinoid residues in pollen were significantly associated with colony growth through time, we constructed linear regression models with relative change as the response and mean clothianidin and thiamethoxam concentrations in pollen in May as the predictor. We also included the relative change in colony pollen coverage over the same time interval as a covariate, to account for the potential that the negative effects of neonicotinoid exposure could be partially offset by the positive effects of increased food supply in corn-rich areas. If the pollen change covariate was not a significant predictor, we dropped the term and refit the model.

Semi-field experiments with closed colonies

To test for a direct association between insecticide contamination of pollen and worker mortality, we conducted a semi-field experiment where small honey bee colonies, enclosed in nucleus hive equipment and denied the ability to freely forage, were fed pollen contaminated with known concentrations of seed treatment neonicotinoids. The colonies were confined in Bee Brief 4-frame nuc boxes (NOD Apiary Products, Frankford, Ontario, Canada), each containing a pollen feeder frame, two frames with drawn wax combs with approximately 100 cm^2 of capped brood for stabilizing the colony, a healthy mated queen (3 – 5 weeks post-mating), and nurse bees shaken from one frame of brood from a healthy colony.

The equipment was weighed prior to adding bees and again after bees were shaken into the box to obtain the net weight of bees per colony. The pollen feeder was made of two 96-well cell culture plates (Biotix #AP-0350-9CU, San Diego, California) affixed to a plastic foundation frame, with 200 g of pollen packed into the plates. Pollen treatments were harvested from different apiaries during May 2 – 19, 2015, to encompass a wide range of neonicotinoid concentrations. All colonies had unlimited access to fresh sugar syrup (1:1 sucrose in water w/w) throughout the experiment. A total of four trials, each including 4 - 7 colonies, were performed (20 colonies total). Each trial also contained a positive control treatment spiked with 400 ppb of clothianidin. Enclosed colonies were kept in a dark room with average daily high temperature of 24.4 °C (daytime) and low temperature of 19.5 °C (nighttime) for 96 hours. Dead bees in the boxes were counted and pollen consumption was determined by weight. Five grams of pollen was sampled at the conclusion of the experiment for pesticide residue analysis. Pearson's correlations were performed to evaluate the association between the number of dead bees and neonicotinoid concentrations. Two colonies were found to contain less than 100 g of bees and were excluded from the analysis.

Results

Worker bee mortality

Increased numbers of dead bees at hive entrances were consistently observed around the time when corn was being planted. Worker mortality was significantly and consistently higher during corn planting than the non-planting periods for the same colonies for all years (paired t-test, 2013: $t = 2.62$, $df = 11$, $P = 0.02$; 2014: $t = 3.24$, $df = 23$, $P = 0.004$; 2015: $t = 11.82$, $df = 37$, $P < 0.0001$) (**Fig. 2a**).

Pollen contamination

Analysis of pollen samples showed that clothianidin and thiamethoxam, the insecticides present in corn seed treatments, were consistently the most abundant neonicotinoid insecticides detected in bee-collected pollen in all years, and the detection of these compounds occurred more frequently (Fisher's Exact Test, $P < 0.0001$) and at higher concentrations during corn planting periods (**Table 1, Fig. 2b**). Neonicotinoid insecticides that are not used for corn seed treatments, but applied to other crops in Ohio (imidacloprid, nitenpyram, dinotefuran, and thiacloprid) were detected in pollen samples, but the timing of detection for other neonicotinoids was not related to corn-planting (**Supplemental Material S2**).

The relationship between neonicotinoid concentration in pollen and the area of corn grown within the foraging range was evaluated for 10 apiary sites studied in 2015. During corn planting, pollen collected from sites with more surrounding cornfields contained higher concentrations of clothianidin (Pearson's $r = 0.65$, $P = 0.040$) and thiamethoxam ($r = 0.62$, $P = 0.056$). The sum concentration of clothianidin and thiamethoxam together was significantly correlated with cornfield area ($r = 0.68$, $P = 0.030$). No correlation between cornfield area and clothianidin or thiamethoxam concentrations were detected outside the planting period ($P > 0.4$).

Relationship between pollen neonicotinoid concentration and worker mortality

To determine if there was any interactive effect between insecticide exposure and cornfield area in the landscape, we further tested for correlations between the number of dead bees and clothianidin and thiamethoxam concentrations in pollen on the same sampling dates for each apiary along a gradient of corn intensity in 2015. Positive correlations between the insecticide concentrations and mortality were detected at sites with more corn fields (> 30%

within 2-km radius from the apiary) in the surrounding landscape (**Table 2**).

Mortality increased linearly with increasing neonicotinoid concentrations at all sites and there is a lagged mortality response to insecticide exposure at half the sites (Table 2). Further, the **mortality rate peaked during or shortly after the planting period as indicated by a pulse intervention term in the time series model.** Differences in mortality rates between planting and non-planting periods were indicated by a linear mortality response to insecticide concentrations and possibly a pulse intervention rather than a step intervention.

Including the other insecticides to the analyses did not improve the fit of the time series intervention model at any site in 2015. I would recommend that the paper include several time series plots of mortality rates and neonicotinoid concentrations for several sites and years in relation to the planting periods.

Insecticide residues in in-hive samples

The total clothianidin and thiamethoxam concentrations in bee bread were generally low before planting (< 13.5 ng/g, mean 4.0 ng/g). The levels increased in samples collected during corn-planting (4.8 - 42.3 ng/g, mean 21.6 ng/g) and immediately after planting (4.5 - 60.8 ng/g, mean 23.6 ng/g). Total clothianidin and thiamethoxam concentrations in stored pollen collected two weeks after planting returned to a lower level (< 11.6 ng/g) except for a sample collected at TV (35.76 ng/g), averaging at 11.6 ng/g for this sampling time. Mean concentrations differed significantly among samping times (one-way ANOVA $F_{(3, 24)} = 3.23$, $P = 0.04$) with the lowest concentration level in bee bread sampled before planting.

Neonicotinoid concentrations for in-hive stored pollen were compared with concentrations detected in corbicular pollen trapped at the same apiaries for the four sampling periods. A significant correlation between the two was detected immediately post-planting ($r = 0.87$, $P = 0.0098$) but not in other sampling periods (Fig. 3).

Concentrations of clothianidin and thiamethoxam in honey were low (< 0.76 ng/g total neonicotinoid, Supplemental Materials S2), though there were more positive detections for honey sampled during and after corn-planting, the mean values did not differ significantly across sampling time.

Closed colony experiments

At the end of the four-day trials, the worker bee mortality was positively and significantly correlated with the total concentration of clothianidin and thiamethoxam in pollen fed to the bees (Pearson's $r = 0.81$, $P < 0.0001$; Fig. 4).

Post-planting colony development.

To address the question of whether seed treatment neonicotinoid exposure in May had long term consequences for colony growth, we tracked six hive health metrics (adult bees, pollen stores, nectar stores, open brood, and capped brood, measured by frame area) at four time points (April, May, June, and August) in 2015. Neonicotinoid exposure in May was correlated with a reduction in the relative population growth of the hive (as measured by area of bees and seams of bees) over the earliest time interval (late April to late May; area of bees: $t = -3.61$, $P = 0.01$;

seams of bees: $t = -2.50$, $P = 0.04$). However, there may have been a recovery in the second time interval, from late May to late June, as hives exposed to greater neonicotinoid levels in May had a larger increase in adult bee population during this time (for seams of bees only; $t = 2.47$, $P = 0.04$). Finally, in the third time interval, from late June to early August, hives located in areas where neonicotinoid exposure during planting was greatest had more stored pollen ($t = 3.35$, $P = 0.01$). The area sown in corn surrounding apiaries was also positively correlated with increases in stored pollen (Pearson's $r = 0.79$, $P = 0.007$) and honey ($r = 0.68$, $P = 0.03$) during the summer period.

Of the 38 colonies monitored, one colony died in late summer and three were relocated to another location and were excluded from monitoring over winter. A total of 34 colonies were prepared for overwintering at the end of September 2015 and 31 of those colonies (91%) were still alive at the end of March, 2016, although one of the surviving colonies was queenless. No significant correlation was observed between survival over winter and the level of corn seed treatment neonicotinoids in pollen or percent corn area in the surrounding landscape across the 10 apiaries (Spearman's rank correlation tests, $P > 0.36$ for all tests).

Discussion

Mortality, exposure, and corn planting

For three years, we consistently observed elevated mortality in adult honey bee workers during corn planting. This pattern of mortality coincided with our finding that clothianidin and thiamethoxam, the insecticidal component in corn seed treatment, were detected more frequently and at higher concentrations in pollen collected by honey bees during corn planting. Additionally, our study demonstrated that seed treatment insecticides can consistently be detected in bee-collected pollen during planting, indicating that the release of seed treatment particles during corn planting is ubiquitous and that released particles are subject to aerial transport, in agreement with previous studies (Krupke et al. 2012, Schaafsma et al. 2015).

Together, these lines of evidence strongly indicate a causal connection between elevated honey bee mortality and seed treatment insecticides emitted during planting. This conclusion is further corroborated by recent work in Italy, where reports of honey bee mortality during corn planting have decreased significantly since the suspension of use of neonicotinoid seed treatments in corn (Sgolastra et al. 2017). Deliberative Process / Ex. 5

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One inconsistency must be considered, though. The concentrations of neonicotinoid insecticides detected in pollen samples were below those that would be expected to cause acute mortality, yet adult mortality was observed in free-flying colonies observed during corn planting and in confined colonies fed pollen collected during corn planting. Based on a range of acute oral LD50s for adult workers of 1.11 - 6.76 ng/bee (Laurino et al. 2013) and predicted pollen consumption of nurse bees 6.5 mg/bee/day (Rortais et al. 2005), substantial mortality would only be expected at concentrations greater than 171 µg/kg in pollen. However, neonicotinoid residues detected in bulk pollen samples may not meaningfully reflect doses received by individual bees

(Sponsler and Johnson 2017). For example, a bulk pollen concentration of 20 μg/kg clothianidin could reflect a uniform distribution of insecticide or it could reflect a skewed distribution in which one or a few pollen pellets carry very high concentrations while the rest of the pollen is relatively uncontaminated. These two distributions would have the same mean concentration, but would result in different effects on the colony. Given the observed pattern of mortality we can infer that neonicotinoid contamination is not evenly distributed throughout all pollen since an increase in bee mortality is reliably observed.

Post-planting colony development

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Deliberative Process / Ex. 5 While high levels were detected in bee bread and honey sampled immediately after corn planting, levels in bee bread and honey declined to the pre-planting level the following week for most sites. Contaminated food stored inside the hives were likely consumed or diluted to lower concentration as uncontaminated pollen and nectar were brought in after planting.

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The sub-lethal effects of the exposure remain unclear and require further investigation. The sudden loss of adult bees occurred at a critical time when colony populations were building up for early-summer honey production and smaller colonies may not be able to recover from a loss of adult workers (Khoury et al. 2013). Beekeepers that depend on early spring buildup to make splits and sell nucleus colonies could be especially hard hit by a loss of bees during corn planting. It is also possible that exposure to neonicotinoids during corn planting could affect queen quality as the planting season coincides with queens rearing for the swarm season (Tsvetkov et al. 2017). Clothianidin and thiamethoxam can also affect honey bee's immunity against viral diseases (Di Prisco et al. 2013) or reduce survival when colonies are under nutritional stress (Tosi et al. 2017).

Conclusion

Our study confirms that seed treatment insecticides are released during corn planting and that these insecticides contaminate pollen collected by bees. Honey bee colonies experience elevated adult mortality due to seed treatment insecticide exposure. **Deliberative Process / Ex. 5**

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Table 1. Frequency of positive detections (Det./n, n = total number of samples) of clothianidin and thiamethoxam residues, and range of concentration when detected, in bee-collected pollen. T-tests assuming unequal variance were performed to compare the means of planting vs. non-planting periods.

		Det./n, range (µg/kg)		Mean±se (µg/kg)		
Year	Residues	Planting	Non-planting	Planting	Non-planting	t-test
2013	Clothianidin	12/12 (100%) 4.8 – 35.5	2/18 (11%) 3.9 – 6.9	16.63 ± 2.96	0.57 ± 0.40	t = 5.38, df = 11 **P = 0.0002**
	Thiamethoxam	8/12 (67%) 1.6 – 9.1	1/18 (6%) 2.2	3.67 ± 1.01	0.12 ± 0.12	t = 3.50, df =11 **P = 0.0048**
	Total	12/12 (100%) 4.8 – 44.6	3/18 (17%) 2.2 – 6.3	20.3 ± 3.75	0.69 ± 0.41	t = 5.19, df = 11 **P = 0.0003**
2014	Clothianidin	5/18 (28%) 12.0 – 18.4	0/55 (0%) N/A	4.15 ± 1.64	0	t = 2.53, df = 17 **P = 0.0219**
	Thiamethoxam	5/18 (28%) 5.6 – 9.3	0/55 (0%) N/A	1.94 ± 0.78	0	t = 2.49, df = 17 **P = 0.0233**
	Total	8/18 (44%) 5.6 – 21.1	0/55 (0%) N/A	6.09 ± 1.89	0	t = 3.24, df = 17 **P = 0.0048**
2015	Clothianidin	30/30 (100%) 2.2 – 91.9	33/60 (55%) 1.2 – 19.5	17.18 ± 3.43	2.89 ± 0.55	t = 4.11, df = 31 **P = 0.0001**
	Thiamethoxam	27/30 (100%) 1.4 – 46.5	33/60 (55%) 1.1 – 14.2	7.43 ± 1.79	2.20 ± 0.44	t = 2.83, df = 33 **P = 0.0039**
	Total	30/30 (100%) 3.6 – 138.4	41/60 (68%) 1.2 – 29.9	24.61± 4.87	5.09 ± 0.85	t = 3.95, df = 31 **P = 0.0002**

Table 2. Interactive effect of site & pollen insecticide concentrations on mortality
Summary of Pearson's correlation test between same-day data of worker mortality and neonicotinoid (clothianidin and thiamethoxam) concentrations in bee-collected pollen for the apiaries monitored in 2015. Sites are presented in the order of corn area (in %) within a 2 km radius centering the apiary.

Site	% corn	r	P	Time series intervention model
DS	1	0.54	0.1371	Mortality = 0.05+0.1396* thiamethoxam_lag1, R2=0.78
SC	8	0.47	0.2015	Mortality = 0.27+0.0322* thiamethoxam+ (0.0147-0.0147*ISC3-0.0118*ISC4-0.0122*ISC17-0.0133*ISC48)*clothianidin+ 0.36*I128, R2=0.52
MB	19	0.44	0.2419	Mortality = 0.03+0.0388*clothianidin+ 0.0259* clothianidin _lag1+0.25*I131, R2=0.60
BR	22	0.31	0.4121	Mortality = 0.34+0.0287*clothianidin*IBG59+ 0.58*I122-0.23I>145, R2=0.67
IB	22	0.49	0.1823	Mortality = 0.03+0.0632*clothianidin+ 0.29*I>142, R2=0.48
WB	30	0.38	0.318	Mortality = 0.22+0.0225*clothianidin_lag3+ 0.0131* thiamethoxam+0.23*IWB13-0.26*I120, R2=0.42
HR	30	0.94	0.0002	Mortality = 0.24+0.0526*thiamethoxam-0.19*IHR5-0.16*IHR6-0.14*HR20, ρ=0.21,R2=0.85
TV	31	0.87	0.0023	Mortality = 0.05+0.0038*clothianidin+ 0.0089*clothianidin_lag1+ 0.0350*thiamethoxam+0.16*ITV62, R2=0.96
MO	39	0.92	0.0004	Mortality = 0.30+(0.0254+0.0122*IMM39)* clothianidin+0.0123*clothianidin_lag1+ 0.16*I131-0.17*IMM16-0.35*IMM39, R2=0.76
FSR	49	0.82	0.0071	Mortality = 0.03+0.0076*clothianidin+ 0.0114* thiamethoxam_lag1+0.28*I143, R2=0.87

Figure 1. Seed treatments are applied to seeds as flowable solids that dry to form a coating. In corn, this coating results in visibly patchy coverage of the seed (a). The seed treatment forms particles of varying size on the surface of the seed as captured using scanning electron microscopy (SEM) (b). The striated surface visible in the center of the micrograph is the seed surface. Particles of the seed treatment coating are then emitted as planter dust during the sowing process (c). Macrophotography was performed by M. Spring, and SEM preparation by K. Kaszas.

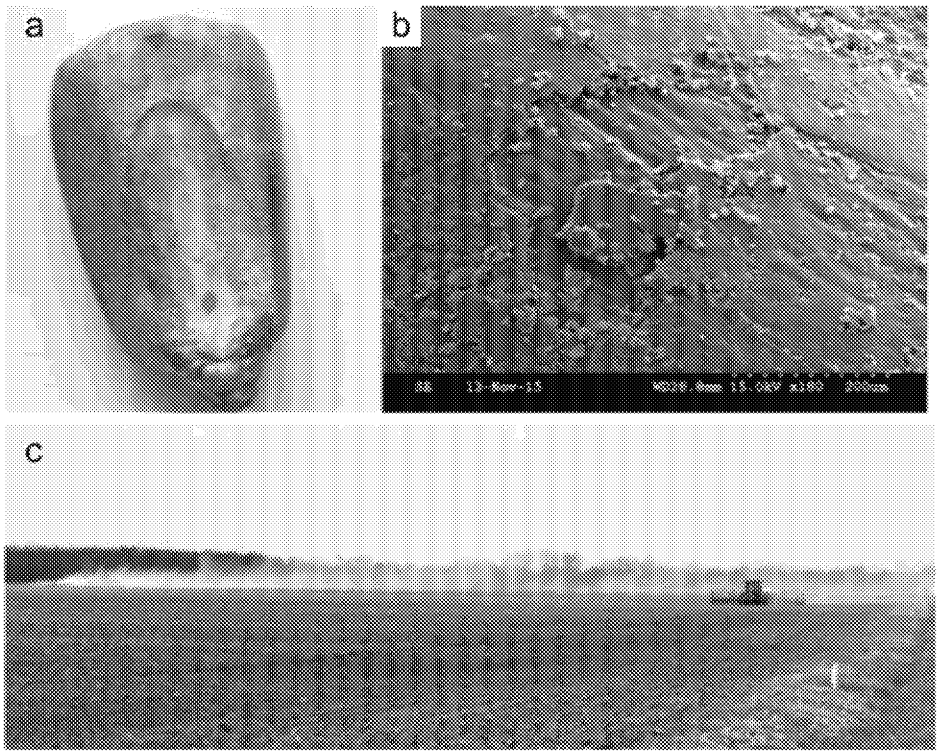

Figure 2. Honey bee worker mortality (a) and neonicotinoid (clothianidin and thiamethoxam) concentrations detected in pollen samples collected during planting and non-planting periods. Whiskers represent one standard error around the means.

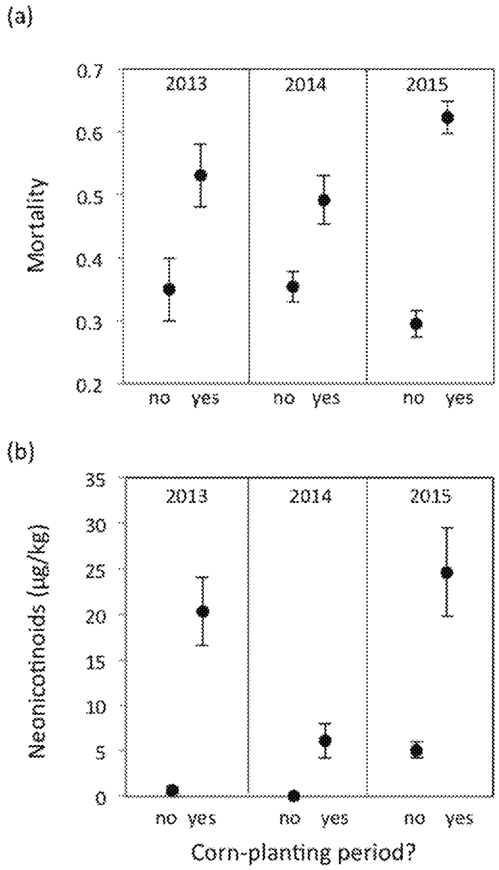

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Figure 3. Summed clothianidin and thiamethoxam concentrations in bee bread and trapped corbicular pollen before, during, and after corn planting.

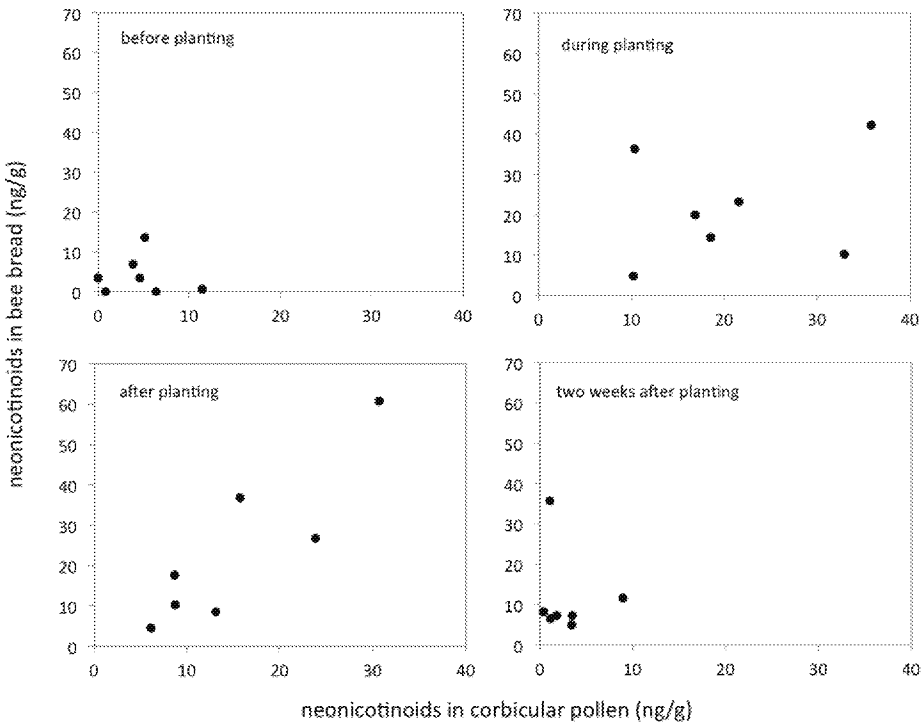

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Figure 4. Number of dead workers and neonicotinoid concentration (ng/g) in enclosed colonies.

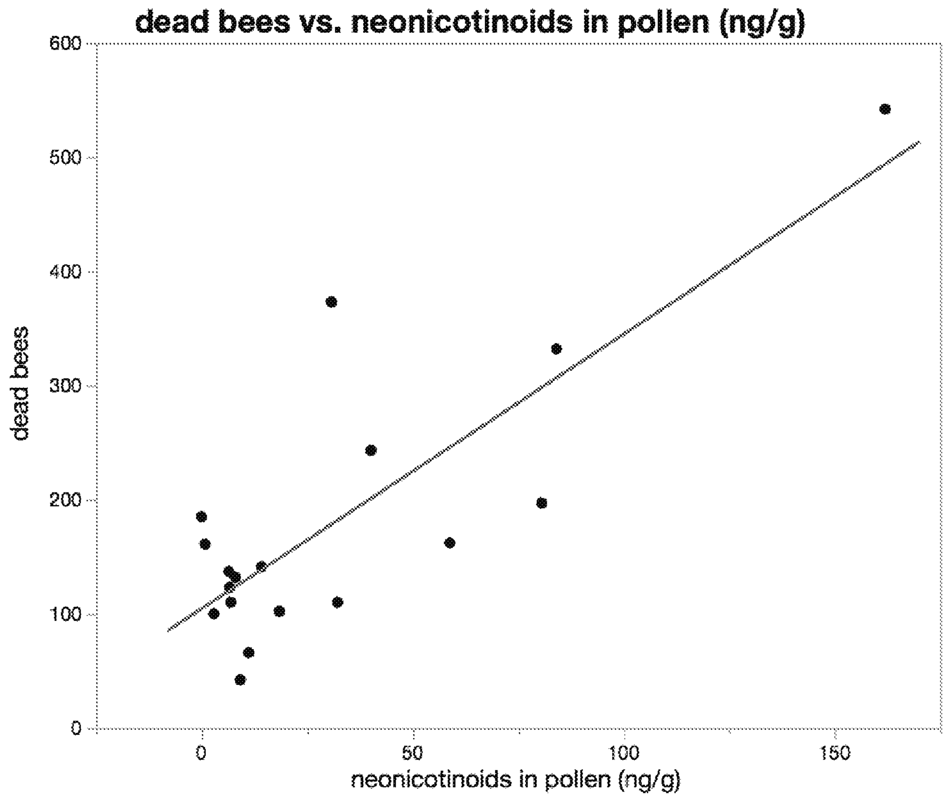

Figure 5: Study apiaries distributed throughout central Ohio. Map of apiaries used in 2014 (yellow), 2015 (blue) and multiple years, including sites used in 2013 (green). Central Ohio landscapes are characterized by field crops (brown) with urban areas (pink and red), forest (green) and pastures (yellow) in the National Landcover Database map (Homer et al. 2015).